AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology

Development of a TaqMan Array Card for Acute-Febrile-Illness Outbreak Investigation and Surveillance of Emerging Pathogens, Including Ebola Virus

Jie Liu,[a] Caroline Ochieng,[b] Steve Wiersma,[c] Ute Ströher,[d] Jonathan S. Towner,[d] Shannon Whitmer,[d] Stuart T. Nichol,[d] Christopher C. Moore,[a] Gilbert J. Kersh,[e] Cecilia Kato,[e] Christopher Sexton,[e] Jeannine Petersen,[e] Robert Massung,[d] Christine Hercik,[f] John A. Crump,[g,h] Gibson Kibiki,[i] Athanasia Maro,[i] Buliga Mujaga,[i] Jean Gratz,[i] Shevin T. Jacob,[j] Patrick Banura,[k] W. Michael Scheld,[a] Bonventure Juma,[l] Clayton O. Onyango,[l] Joel M. Montgomery,[l,m] Eric Houpt,[a] Barry Fields[l,m]

Division of Infectious Diseases and International Health, University of Virginia, Charlottesville, Virginia, USA[a]; Center for Global Health Research, Kenya Medical Research Institute, Nairobi, Kenya[b]; Centers for Disease Control and Prevention, Center for Global Health, Division of Global Health Protection, Dar es Salaam, Tanzania[c]; Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases, Division of High Consequence Pathogens and Pathology, Atlanta, Georgia, USA[d]; Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases, Division of Vector Borne Diseases, Atlanta, Georgia, USA[e]; Georgetown University, Washington, DC, USA[f]; Division of Infectious Diseases and International Health, Duke University, Durham, North Carolina, USA[g]; Centre for International Health, University of Otago, Dunedin, New Zealand[h]; Kilimanjaro Clinical Research Institute, Moshi, Tanzania[i]; Division of Allergy and Infectious Diseases, University of Washington, Seattle, Washington, USA[j]; Masaka Regional Referral Hospital, Masaka, Uganda[k]; Centers for Disease Control and Prevention, Center for Global Health, Division of Global Health Protection, Nairobi, Kenya[l]; Centers for Disease Control and Prevention, Center for Global Health, Division of Global Health Protection, Atlanta, Georgia, USA[m]

Acute febrile illness (AFI) is associated with substantial morbidity and mortality worldwide, yet an etiologic agent is often not identified. Convalescent-phase serology is impractical, blood culture is slow, and many pathogens are fastidious or impossible to cultivate. We developed a real-time PCR-based TaqMan array card (TAC) that can test six to eight samples within 2.5 h from sample to results and can simultaneously detect 26 AFI-associated organisms, including 15 viruses (chikungunya, Crimean-Congo hemorrhagic fever [CCHF] virus, dengue, Ebola virus, Bundibugyo virus, Sudan virus, hantaviruses [Hantaan and Seoul], hepatitis E, Marburg, Nipah virus, o'nyong-nyong virus, Rift Valley fever virus, West Nile virus, and yellow fever virus), 8 bacteria (*Bartonella* spp., *Brucella* spp., *Coxiella burnetii*, *Leptospira* spp., *Rickettsia* spp., *Salmonella enterica* and *Salmonella enterica* serovar Typhi, and *Yersinia pestis*), and 3 protozoa (*Leishmania* spp., *Plasmodium* spp., and *Trypanosoma brucei*). Two extrinsic controls (phocine herpesvirus 1 and bacteriophage MS2) were included to ensure extraction and amplification efficiency. Analytical validation was performed on spiked specimens for linearity, intra-assay precision, interassay precision, limit of detection, and specificity. The performance of the card on clinical specimens was evaluated with 1,050 blood samples by comparison to the individual real-time PCR assays, and the TAC exhibited an overall 88% (278/315; 95% confidence interval [CI], 84% to 92%) sensitivity and a 99% (5,261/5,326, 98% to 99%) specificity. This TaqMan array card can be used in field settings as a rapid screen for outbreak investigation or for the surveillance of pathogens, including Ebola virus.

Fever is a symptom common to a wide variety of infectious diseases, including some of the leading causes of death in sub-Saharan Africa (SSA). Many etiologic studies have been performed for respiratory infections, diarrheal illness, and meningitis (1, 2). However, the incidence and etiology of undifferentiated fever are less clear (3). Most research has examined individual agents such as *Plasmodium*, *Salmonella*, and specific zoonotic or arboviral pathogens (4–6) by utilizing blood culture (7) or a complex mixture of rapid, serologic, culture, and molecular assays and algorithms to determine an etiologic agent (8).

We describe our initial development and validation of a TaqMan array card (TAC) that uses quantitative reverse transcription-PCR (qRT-PCR) for the simultaneous detection of 15 viruses, 8 bacteria, and 3 protozoa of particular relevance to SSA (5, 9–13), with the intended use for outbreak investigation and acute febrile illness (AFI) surveillance. Previous TAC assays have been developed for respiratory diseases, enteric diseases, and etiologies of neonatal sepsis (14–16), and we have shown their robust and comparable performance across several countries (17). Once developed, TaqMan array cards are stable at 4°C for 2 years, can be shipped at ambient temperature, and minimize several cumbersome steps in the field, such that they are as easy to perform as individual quantitative PCR (qPCR) assays.

This work was primarily a development exercise since clinical

Received 19 August 2015 **Returned for modification** 9 September 2015
Accepted 28 September 2015

Accepted manuscript posted online 21 October 2015

Citation Liu J, Ochieng C, Wiersma S, Ströher U, Towner JS, Whitmer S, Nichol ST, Moore CC, Kersh GJ, Kato C, Sexton C, Petersen J, Massung R, Hercik C, Crump JA, Kibiki G, Maro A, Mujaga B, Gratz J, Jacob ST, Banura P, Scheld WM, Juma B, Onyango CO, Montgomery JM, Houpt E, Fields B. 2016. Development of a TaqMan array card for acute-febrile-illness outbreak investigation and surveillance of emerging pathogens, including Ebola virus. J Clin Microbiol 54:49–58. doi:10.1128/JCM.02257-15.

Editor: A. J. McAdam

Address correspondence to Eric Houpt, erh6k@virginia.edu, or Barry Fields, bsf2@cdc.gov.

E.H. and B.F. contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.02257-15.

validation was limited for many rare pathogens. However, we propose that this diagnostic tool can potentially screen for Ebola virus disease (18) or indicate other etiologies of fever such as malaria, dengue, or typhoid. Indeed the slow recognition of the Ebola outbreak in Guinea was largely due to delays in laboratory diagnosis and confirmation and to the inability to perform such testing in the region (9).

MATERIALS AND METHODS

TAC design. Primers and probes for 26 pathogens and 2 extrinsic controls were included on the TaqMan array card. Primer and probe sequences were adapted from published assays whenever possible (Table 1). The assays were evaluated *in silico* with BLAST, Clustal, and Primer Express (Life Technologies, Carlsbad, CA). When needed, slight modifications were made to primer or probe length to optimize performance under universal cycling conditions, and modified versions were tested in parallel with the original assays. Most assays were performed in duplicate to maximize sensitivity as laid out in Fig. 1. Assays were validated on plates using the TaqMan array card universal formula, which is a final primer concentration of 900 nM and a probe concentration of 250 nM. This custom TaqMan array card was manufactured by Life Technologies.

Specimens. Analytical specimens included genomic materials, cultured organisms, and *in vitro* transcripts for several RNA targets, as indicated in Table S1 in the supplemental material. In addition, a total of 1,050 clinical samples were collected through several studies conducted across Africa (Table 2), including 362 archived samples selected based on their likelihood for being positive for some of the pathogens on the TaqMan array card and 688 blood specimens from disease surveillance representing the AFI cases in relevant regions in Tanzania. These samples were frozen at −70°C prior to testing. Briefly, the archived samples included the following: (i) 105 serum samples (166 μl extracted) from outbreak investigations and previous studies at the U.S. Centers for Disease Control and Prevention (CDC)-Kenya from 2008 to 2014, (ii) 49 RNA samples (100 μl extracted) obtained from the ELWA III hospital laboratory in Monrovia, Liberia, during the Ebola virus outbreak in 2014, (iii) 186 whole blood samples (1 to 2.5 ml extracted) obtained at two clinical sites from patients presenting with moderate to severe febrile illness in Kilombero, Tanzania, in 2014, (iv) 16 whole blood specimens (2.5 ml extracted) obtained from inpatients at Kilimanjaro Christian Medical Centre and Mawenzi Regional Hospital, Moshi, Tanzania (10), and (v) 4 whole blood samples (1 ml extracted) known to be positive for *Salmonella enterica* by culture obtained from adult patients with severe sepsis enrolled in a fluid resuscitation study in Uganda and 2 whole blood samples (2.5 ml extracted) that were blood culture positive for *Salmonella enterica* from the clinical laboratory at the University of Virginia. All of the blood samples were collected in Vacutainer EDTA tubes. All tests were performed with institutional approvals as follows: KEMRI ERC-SSC 1899 (SSC 932), Duke University Health System Institutional Review Board protocol 8400-06-4R0, Kilimanjaro Christian Medical Centre Research Ethics Committee protocol 136, Tanzania National Institutes for Medical Research National Research Ethics Coordinating Committee protocol NIMR/HQ/R.8a/Vol. IX/439 and 1735, Uganda National Council for Science and Technology (UNCST HS 419), Centers for Disease Control and Prevention Institutional Review Board protocol 6567, and the University of Virginia HSR 13393 and 17391, respectively.

Combined positive controls. Two combined positive controls, one for DNA targets and one for RNA targets, were designed as previously described (16, 19). Plasmids were synthesized by GeneWiz (South Plainfield, NJ) and were used directly as DNA or *in vitro* transcribed as RNA.

Nucleic acid extraction from blood samples. For the archived CDC-Kenya clinical samples in the first study (Table 2), 166 μl of each sample was processed in a KingFisher ML extraction platform (Thermo Scientific, Waltham, MA) using a MagMAX nucleic acid isolation kit (Life Technologies, Carlsbad, CA). Briefly, 166 μl of sample was mixed with 433 μl of lysis-binding solution and was then washed once with 600 μl wash solution 1 and twice with 450 μl wash solution 2 and was eventually eluted in 200 μl elution buffer. For the samples from the second study, viral RNA was extracted from 100 μl of blood and was eluted with 90 μl of elution buffer on the MagMAX Express-96 deep-well magnetic particle processor (Life Technologies) using the MagMAX pathogen RNA/DNA kit following the manufacturer's instructions. For the analytical specimens and clinical specimens from the third, fourth, fifth, and sixth studies, total nucleic acid (TNA) was extracted using a High Pure viral nucleic acid large volume kit (Roche) by following the manufacturer's instructions. Extrinsic controls, 10^6 copies of phocine herpesvirus (PhHV) and 10^7 MS2 bacteriophage (ATCC, Manassas, VA), were added to each sample during the lysate preparation to evaluate extraction and amplification efficiency. The total nucleic acid was eluted in 100 or 200 μl of elution buffer to accommodate the sample need of running individual real-time PCR (IRTP) for all 26 targets (Table 2). One extraction blank was included with each batch of extraction to monitor lab contamination, and if it was positive for a given target, then the positive results for this target in the entire batch were considered invalid (this only happened once, invalidating the *Salmonella* results of 5 clinical samples).

PCR using TaqMan array cards. All analytical specimens and 1,050 clinical samples were tested with TaqMan array cards. We mixed 46 or 75 μl of total nucleic acid extract with AgPath one-step RT-PCR reagents or with TaqMan fast virus one-step master mix (Life Technologies), respectively, in a 100-μl reaction mixture, as shown in Table 2, and then pipetted the mixture into the inlet port of each channel. Cards were centrifuged (1 min at 1,200 rpm twice) and sealed, and the inlet ports were removed following the manufacturer's instructions. Cards were run on the ViiA 7 real-time PCR system (Life Technologies) using PCR cycling conditions comprising 10 min at 50°C and 20 s at 95°C followed by 45 two-step cycles of 3 s at 95°C and 30 s at 60°C. A sample is called positive when any of the duplicate reactions yield amplification (quantification cycle $[C_q] < 45$).

Performance on analytical specimens. Linearity was tested with a 10-fold serial dilution of combined positive controls. For limit of detection (LOD), intra-assay precision, and interassay precision, positive materials (either genomic material, organisms, or *in vitro* transcripts as indicated in Table S1 in the supplemental material) were spiked into lysis buffer and added to blood samples from healthy donors (2.5 ml blood per sample). Nucleic acid was extracted and assayed in the card, as described above, with an elution volume of 100 μl and a sample volume of 75 μl. Intra-assay precision was tested with eight repeats on one single TAC with pooled extracts from 8 identical 2.5-ml spiked samples. Interassay precision was tested with 10 identically spiked samples that were extracted and assayed over 5 days. Limit of detection was estimated as the lowest concentration at which the target could be detected in all 10 spiked samples. Matrix inhibition was evaluated with extrinsic controls spiked into each clinical sample type (serum, whole blood, and plasma) in the Tanzania surveillance study, where 688 samples were in various forms, including 55 whole blood samples (0.7 to 2.5 ml), 419 serum samples (0.25 to 1.5 ml), and 214 plasma samples (1 ml). For genus-specific bacterial and protozoan assays, multiple species were assayed as listed in Table S2 in the supplemental material. A panel of commonly found bloodstream pathogens were also spiked at a concentration of 10^7 copies per extraction and were tested to evaluate the specificity of each assay, including *Acinetobacter baumannii*, *Cryptococcus neoformans*, cytomegalovirus, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Toxoplasma gondii*.

Performance on clinical specimens. Comparison of the TAC was made against individual real-time PCR (IRTP), with the cognate assays tested on plates with 2 to 5 μl of nucleic acid, the same PCR master mix, and the same PCR conditions. The number of samples that were tested for each pathogen was listed in Table 2. All samples that tested positive on TAC for a given target were evaluated for the corresponding targets on

TABLE 1 Primer and probe sequences for the TaqMan array card assays

Pathogen	Target	Sequence (5′–3′)[a]	Reference
Viruses			
Chikungunya	NSP4	F: TCACTCCCTGYTGGACTTGATAGA R: TTGACGAACAGAGTTAGGAACATACC P: AGGTACGCGCTTCAAGTTCGGCG	Modified (34)
CCHF	NP	F: CAAAGAAACACGTGCCGCTT R: ATTCACCTCGATTTTGTTTTCCAT P: ACGCCCACAGTGTTCTCTTGAGTGTTAGCA	
Dengue	3′NC	F: GGATAGACCAGAGATCCTGCTGT R: CATTCCATTTTCTGGCGTTC R: CAATCCATCTTGCGGCGCTC P: CAGCATCATTCCAGGCACAG	(35)
Ebola Zaire	NP	F: TGGAAAAAACATTAAGAGAACACTTGC R: AGGAGAGAAACTGACCGGCAT P: CATGCCGGAAGAGGAGACAACTGAAGC	(28)
Bundibugyo	VP40	F: MGCATCRTAYACCATCACTCA R: SCCAGGACCAAGTCGRTTGA P: TTTGGCAAAACCTCMAATCC	
Sudan	NP	F: GCCATGGITTCAGGTTTGAG R: GGTIACATTGGGCAACAATTCA P: ACGGTGCACATTCTCCTTTTCTCGGA	(13)
Hantavirus	NP	F: CATGGCWTCHAAGACWGTGGG R: TTKCCCCAGGCAACCAT P: CAATCAATGGGRATACAACTGG	Modified (36)
Hepatitis E	ORF3	F: GGTGGTTTCTGGGGTGAC R: AGGGGTTGGTTGGATGAA P: TGATTCTCAGCCCTTCGC	(37)
Marburg	VP40	F: GGACCACTGCTGGCCATATC R: GAGAACATITCGGCAGGAAG P: AAAGTCCCAGAGAAGACA	Modified (20)
Nipah	NP	F: CTGGTCTCTGCAGTTATCACCATCGA R: ACGTACTTAGCCCATCTTCTAGTTTCA P: CAGCTCCCGACACTGCCGAGGAT	(38)
ONNV	E1	F: GCAGGGAGGCCAGGACAGT R: GCCCCTTTTTCYTTGAGCCAGTA P: TGTATTGCTCCTGCCGCTGG	Modified (39)
Rift Valley fever	L	F: TGAAAATTCCTGAGACACATGG R: ACTTCCTTGCATCATCTGATG P: CACAAGTCCACACAGGCCCCTTACAT	Modified (40)
West Nile	3′NC	F: CAGACCACGCTACGGCG R: CTAGGGCCGCGTGGG P: TCTGCGGAGAGTGCAGTCTGCGAT	(41)
Yellow fever	RdRp	F: GGGAAAACTCAGGAGGAGGA F: GGGAGAATTCRGGGGGAGGA R: AAGGTCTGCCTCTGTGATGC P: TCAGAGACCTGGCTGCAATGGATGGT	Modified (42)
Bacteria			
Bartonella spp.	ssrA	F: GGCTAAATIAGTAGTTGCAAAYGACA R: GCTTCTGTTGCCAGGTG P: ACCCCGCTTAAACCTGCGACG	Modified (43)
Brucella spp.	IS711	F: GCTTGAAGCTTGCGGACAGT R: GGCCTACCGCTGCGAAT P: AAGCCAACACCCGGCCATTATGGT	(44)
Coxiella burnetii	IS1111	F: CCGATCATTTGGGCGCT R: CGGCGGTGTTTAGGC P: TTAACACGCCAAGAAACGTATCGCTGTG	(45)
Leptospira spp.	LipL32	F: CCCTAIGGATCTGTRATCAACTA R: GAACTCCCATTTCAGCGATT P: AAAGCCAGGACAAGCGCCG	Modified (46)
Rickettsia spp.	23S	F: AGCTTGCTTTTGGATCATTTGG R: TTCCTTGCCTTTTCATACATCTAGT P: CCTGCTTCTATTTGTCTTGC	Modified (47)

(Continued on following page)

TABLE 1 (Continued)

Pathogen	Target	Sequence (5′–3′)	Reference
Salmonella enterica	ttr	F: CTCACCAGGAGATTACAACATGG R: AGCTCAGACCAAAAGTGACCATC P: CACCGACGGCGAGACCGACTTT	(48)
Salmonella Typhi	STY0201	F: CGCGAAGTCAGAGTCGACATAG R: AAGACCTCAACGCCGATCAC P: CAGCCTGCTCCAGAACA	Modified (49)
Yersinia pestis	Caf1	F: CCACTGCAACGGCAACTCTT R: TGTAATTGGAGCGCCTTCCT P: TTGAACCAGCCCGCATCACTCTTACA	
Protozoa			
Leishmania spp.	18S	F: AAGTGCTTTCCCATCGCAACT R: GACGCACTAAACCCCTCAA P: CGGTTCGGTGTGTGGCGCC	Modified (50)
Plasmodium spp.	18S	F: GCTCTTTCTTGATTTCTTGGATG R: AGCAGGTTAAGATCTCGTTCG P: CACGAACTAAAAACGGCCAT	Modified (51)
Trypanosoma brucei	18S	F: CGCCAAGCTAATACATGAACCAA R: TAATTTCATTCATTCGCTGGACG P: CTTGTGTTTACGCACTTGTC	Modified (52)

[a] F, forward primer; R, reverse primer; P, TaqMan MGB probe.

96-well or 384-well plates, and a subset of negative samples (to the extent that specimen was available) was also retested on plates. Positive samples from studies 4, 5, and 6 (Table 2) were further confirmed by amplicon sequencing with the assays listed in Table S3 in the supplemental material.

Statistics. Quantification cycle (C_q) values were compared with the Mann-Whitney U test between sequence-confirmed and -unconfirmed samples and between IRTP-confirmed and -unconfirmed samples, among different sample types. C_q values obtained from the TAC and IRTP

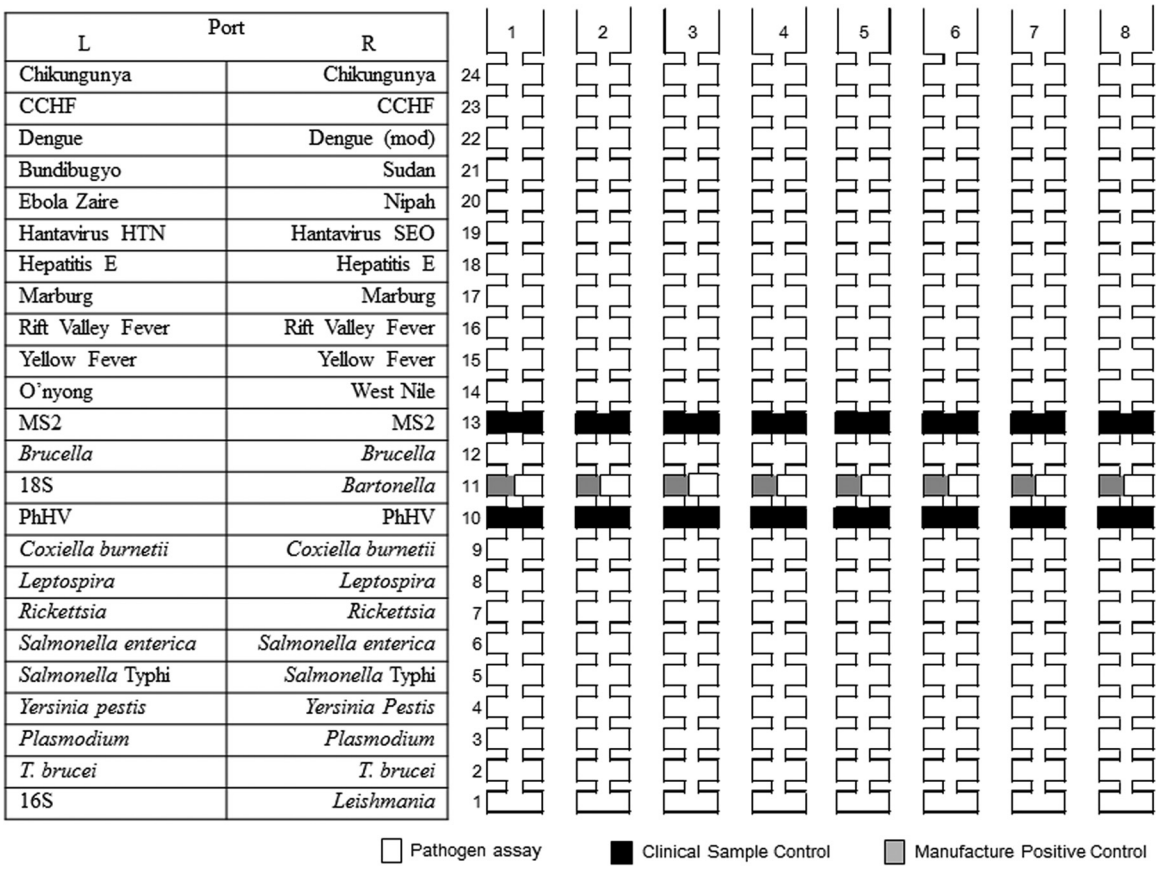

FIG 1 Configuration of the TaqMan array card for detection of the agents causing acute febrile illness.

TABLE 2 Clinical specimens used in this study and the corresponding sample processing methods[a]

Study ID	Selection rationale	No. of samples	Region	Sample type (no.)	Extraction		TAC	
					Method	Elution vol (μl)	Enzyme	Sample vol (μl)
1	Outbreak investigation	105	Kenya (various)	Serum	MagMAX	200	AgPath	46
2	Ebola virus outbreak	49	Monrovia, Liberia	Whole blood	MagMAX	90	AgPath	46
3	Hospitalized AFI patients	186	Kilombero, Tanzania	Whole blood	High Pure	200	AgPath	46
4	Hospitalized AFI patients	16	Moshi, Tanzania	Whole blood	High Pure	100	TaqMan fast virus kit	75
5	Patients presenting fever with blood culture positive for *Salmonella*	6	Uganda (4), Virginia (2)	Whole blood	High Pure	100	TaqMan fast virus kit	75
6	AFI cases	688	Tanzania (various)	Serum (419), plasma (214), whole blood (55)	High Pure	100	TaqMan fast virus kit	75

[a] No difference in detection of external controls (MS2 and PhHV) was observed with the deviations in extraction methods and PCR reagents (data not shown).

for the same samples were compared with the Wilcoxon signed-rank test. Receiver operating characteristic (ROC) analysis was used to derive C_q cutoffs based on sequencing results as the gold standard. Mean and standard deviation were shown. Correlation was tested by regression analysis using the analysis of variance (ANOVA) test. Two-tailed *P* values were calculated, and values of <0.05 were considered statistically significant. All analyses were performed using SPSS version 22.

RESULTS

Performance on analytical specimens. Each TAC assay detected the relevant species or serovar with 100% specificity using the specificity panel. Assays exhibited a linear relationship between C_q values and quantity (R^2 from 0.994 to 1), high PCR efficiency (91% ± 5%), and robust intra-assay precision and interassay precision. The lower limit of detection was estimated to be 10^4 copies/ml of blood for viral targets, 10^3 copies/ml of blood for bacterial targets, and 10^2 copies/ml of blood for parasitic targets, equivalent to 2 to 200 copies (prior to extraction) per 1-μl reaction mixture. The extrinsic controls MS2 and PhHV were used to assess matrix inhibition and were detected in 91% of the whole blood samples, in >99% of the serum samples, and in >97% of the plasma samples. Surprisingly, whole blood samples yielded lower C_q values for the extrinsic controls than those for serum and plasma but were within 2 C_qs (data not shown). Considering that some of the pathogens interrogated are intracellular, we would recommend using whole blood.

Performance on clinical specimens. We then proceeded to the clinical specimens, sourcing material from several studies in attempts to obtain blood samples that may be PCR positive for dengue, Ebola virus, hepatitis E, Rift Valley fever virus, *Bartonella* spp., *Brucella* spp., *Coxiella burnetii*, *Leptospira* spp., *Rickettsia* spp., *Plasmodium* spp., and *Salmonella enterica*, including serovar Typhi. Among duplicate reactions on the TAC, most (75%) were positive in the two wells while the rest were positive in only one well (usually at higher C_q, averaging 38.7 ± 3.3 versus 25.3 ± 5.3 for duplicate positives; $P < 0.001$). Either was considered positive by TAC. All positive TAC results were tested with plate-based PCR using the cognate assays (Table 3), and negative TAC results were retested to the extent that sample quantity allowed. Compared to the plate-based assays, the overall sensitivity and specificity of TAC were 88% (95% confidence interval, 84% to 92%) and 99% (98% to 99%), respectively. Discrepancies were generally observed at late C_q values. For example, the TAC-positive/IRTP-positive samples for *Brucella* yielded a TAC C_q of 34.2 ± 0.6 while the TAC-positive/IRTP-negative samples yielded 38.3 ± 4.0 ($P < 0.05$) and the TAC-negative/IRTP-positive samples yielded 40.3 ± 2.3 ($P < 0.05$). Likewise, the dengue TAC-positive/IRTP-positive samples yielded a TAC C_q of 24.3 ± 5.6 versus the TAC C_q of TAC-positive/IRTP-negative samples of 38.0 ± 2.3 ($P < 0.05$). In contrast, no difference in C_q was observed between the TAC-positive/IRTP-positive and the TAC-negative/IRTP-positive samples for hepatitis E. Overall, TAC C_qs were higher than the corresponding C_qs obtained from IRTP by an average ΔC_q of 1.8 ± 4.1 ($P < 0.05$) (Fig. 2), which corresponds to approximately 1 log loss in sensitivity.

Sequence confirmation. In order to further confirm the positive TAC results, amplicons of a subset of samples were sent for sequencing. Since TaqMan assays require short amplicons that are not suitable for direct sequencing, primers flanking the targeted regions were designed or adapted from publications to generate longer amplicons (for primers, see Table S3 in the supplemental material). The amplification results with these confirmatory assays correlated well with those of the original TAC assays run on a plate (data not shown). As expected, the C_q values of the samples that were sequence confirmable were significantly lower than the values of those that could not be sequenced. ROC analysis revealed that C_q values of 34.3, 32.8, 35.7, 34.9, and 35.0 or less for dengue, *Plasmodium*, *Rickettsia*, *Brucella*, and *Salmonella enterica* maximized the likelihood of sequence confirmation (Fig. 3); therefore, in our view results below these C_qs can be trusted.

Comparison of TAC results with initial testing. The clinical samples were collected from diverse sites across time and were stored in a range of conditions with limited clinical microbiology; therefore, clinical performance versus culture or other non-PCR method is uncertain. Furthermore, we documented obvious degradation of the specimens by comparing the PCR C_q values of the samples when tested upon initial collection with identical real-time PCR assays (data not shown). That said, we do know that seven whole blood samples were initially positive for *Salmonella enterica* by blood culture, of which TAC identified five (after extraction of various volumes of blood from 166 μl to 2.5 ml; sensitivity, 71%). There were also 30 positive *Salmonella enterica* culture samples that were stored in clot or serum form, of which none were positive by TAC. There were 2 samples originally positive for *Leishmania* by rapid test (rK39), and they were both negative by TAC but positive by IRTP at late C_qs (38 and 42). The only mi-

TABLE 3 Sensitivity and specificity of TAC assays on clinical specimens compared to individual real-time PCR assays[a]

Pathogen	No. of samples				% TAC sensitivity (95% CI)	% TAC specificity (95% CI)	Source(s) of the positives[b]
	IRTP positive and TAC positive	IRTP positive and TAC negative	IRTP negative and TAC positive	IRTP negative and TAC negative			
Chikungunya	0	0	2	216	NA[c]	99 (97–100)	NA
CCHF	0	0	0	218	NA	100 (98–100)	NA
Dengue	37	9	8	203	80 (66–91)	96 (93–98)	Studies 1 and 6
Ebola	49	0	0	266	100 (93–100)	100 (99–100)	Study 2
Bundibugyo	0	0	0	208	NA	100 (98–100)	NA
Sudan	0	0	0	217	NA	100 (98–100)	NA
Hantavirus	0	0	0	209	NA	100 (98–100)	NA
Hepatitis E	32	9	0	206	78 (62–89)	100 (98–100)	Study 1
Marburg	0	0	0	213	NA	100 (98–100)	NA
Nipah	0	0	0	217	NA	100 (98–100)	NA
ONNV	0	0	0	218	NA	100 (98–100)	NA
Rift Valley fever	7	1	0	215	88 (47–100)	100 (98–100)	Study 1
West Nile	0	0	0	180	NA	100 (98–100)	NA
Yellow fever	0	0	0	219	NA	100 (98–100)	NA
Bartonella spp.	1	0	0	217	100 (3–100)	100 (98–100)	Study 6
Brucella spp.	8	6	11	205	57 (29–82)	95 (91–97)	Studies 1 and 6
Coxiella burnetii	4	0	6	216	100 (40–100)	97 (94–99)	Study 6
Leptospira spp.	3	0	11	204	100 (29–100)	95 (91–97)	Studies 3 and 4
Rickettsia spp.	24	2	8	211	92 (75–99)	96 (93–98)	Studies 4 and 6
Salmonella enterica	7	1	5	223	88 (47–100)	98 (95–99)	Studies 1, 5, and 6
Salmonella Typhi	4	1	2	225	80 (28–99)	99 (97–100)	Studies 1, 5, and 6
Yersinia pestis	0	0	0	218	NA	100 (98–100)	NA
Leishmania spp.	0	2	0	215	0 (0–84)	100 (98–100)	Study 1
Plasmodium spp.	102	6	12	143	94 (88–98)	92 (87–96)	Studies 1, 3, 5, and 6
Trypanosoma brucei	0	0	0	179	NA	100 (98–100)	NA
Total	278	37	65	5261	88 (84–92)	99 (98–99)	

[a] No cutoff was applied, and the two tests were run for 45 cycles. A sample was called positive when any of the duplicate reactions yielded amplification ($C_q < 45$).
[b] Studies 1 to 6 are listed in Table 2.
[c] NA, not applicable.

croscopy data we have show that eight samples were positive for *Plasmodium* by microscopy, and all 8 were detected by TAC and IRTP.

DISCUSSION

This work details the development of an integrated TaqMan array card that can be used on blood samples to screen for several infectious etiologies of acute febrile illness. Given that there may be multiple agents contributing to fever, this card may be an efficient tool for pathogen detection using a single reaction.

The pathogens we chose to test on this card were optimized for our intended use of outbreak investigation and of AFI surveillance in Africa (5, 9–11, 13, 20). Depending on demographic and temporal or geographic circumstances, users may prefer to prioritize certain pathogens to be included. Since the format is a modular arrangement of singleplex PCR assays, one could include primer and probe sequences from relevant microbial agents (e.g., *Streptococcus pneumoniae*, *Neisseria meningitidis*, etc.) that have been previously validated in TAC platforms for other syndromes with similar cycling conditions (14–16). In our view, this is an advantage of TAC over conventional multiplex PCR-based platforms (e.g., Biofire's BioThreat panel, FTD Tropical panels). We utilized primer and probe sequences from the published literature wherever possible to leverage the considerable experience of subject matter experts given the many pathogens assayed. All assays revealed excellent analytical performance, and the limit of detection of the assays was estimated to be 10^2 to 10^4 copies/ml of blood, similar to that of the previously reported enteric or respiratory cards.

This limit of detection would be expected to be adequate to detect dengue, chikungunya, Ebola virus, and *Plasmodium* in most patients since these pathogens are usually present at even higher levels (21–24). Indeed, we were able to evaluate the assay on large numbers of specimens that were PCR positive for Ebola, dengue, hepatitis E, and *Plasmodium*, and we observed, as expected, reasonable sensitivities (100%, 80%, 78%, and 94%, respectively) and high specificities (96% to 100%) (Table 3). For these pathogens, where PCR is generally considered a gold-standard diagnostic test (particularly early in illness) (25–27), the performance of the TAC assay is likely to be acceptable. For Ebola, we deliberately used the exact nucleoprotein (NP) RT-PCR assay frequently used during outbreak investigation (28), and TAC revealed 100% sensitivity and 100% specificity versus those of the individual assay on 49 samples with diverse Ebola viral loads and a tight C_q correlation (RT-PCR C_q values ranging from 16 to 37; $R^2 = 0.87$; $P < 0.001$). Forty-five percent of these samples were also positive for *Plasmodium*, reinforcing the value of a multitarget diagnostic tool.

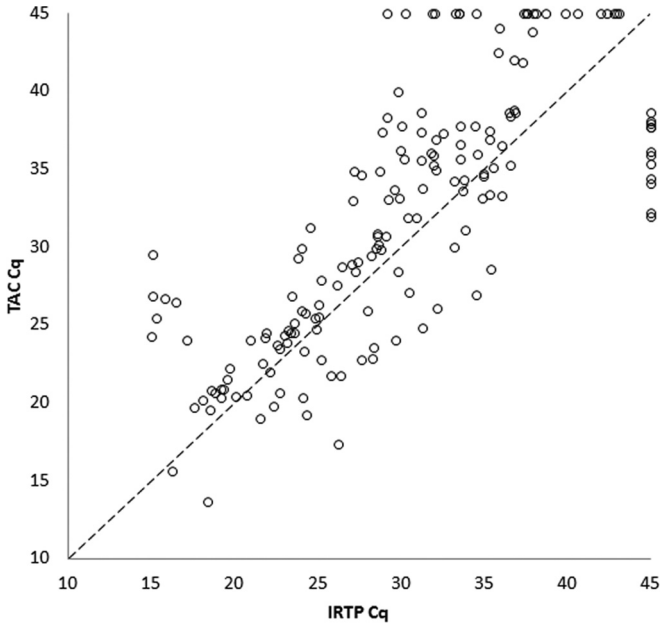

FIG 2 Paired comparison of C_qs from IRTP and TAC reactions. In general, the C_qs exhibited a linear relationship ($R^2 = 0.632$; $P < 0.001$). The results from different targets were pooled and compared with the Wilcoxon signed-rank test for the samples where IRTP and TAC yielded positive results. The difference was within 2 units (IRTP C_q – TAC $C_q = -1.8 \pm 4.1$; $P < 0.001$). The data points at the top and right of the graph represented IRTP-positive/TAC-negative and IRTP-negative/TAC-positive results, respectively. There was no significant difference in the number of samples that were IRTP positive/TAC negative or IRTP negative/TAC positive (chi-square test; $P = 0.205$).

We anticipated that the sensitivity of PCR for bacterial pathogens on a single blood specimen, particularly with TAC and its small sample volumes (up to 0.75 μl nucleic acid in the 1-μl reaction mixture), may be mediocre versus that of culture. Meta-analyses of commercial PCR tests for detecting bacteria in blood from sepsis patients reveal sensitivities of only 61% to 80% versus blood culture (29). This is particularly well known for typhoidal and nontyphoidal *Salmonella enterica*, where in one study the median bacterial burden was 0.3 CFU/ml in blood (30, 31), and *Salmonella* Typhi PCR performed directly on blood has often revealed sensitivity of ~50% versus that of culture (32). Our results with TAC were in keeping with these prior studies (71% sensitivity versus that of culture; >80% sensitivity versus that of IRTP), even though we purposely selected an extraction method that could process up to 2.5 ml of blood to improve sensitivity. Therefore, we acknowledge that this AFI TAC may have limited sensitivity for diagnosis of certain pathogens if it were applied to individual patients using single specimens. However, our main goal was to develop the card for surveillance or outbreak purposes, and in this context one could use TAC to test hundreds of specimens given its high throughput and any limitations in sensitivity could be statistically managed. For example, if one detected by TAC 20 cases of typhoid fever out of 1,000 specimens, then even accepting a 50% sensitivity of PCR versus that of culture, the predicted 95% confidence interval of typhoid prevalence would be 2.8% to 5.4%. In other words, testing larger numbers of specimens for surveillance and outbreak investigation could lead to tight estimates even with imperfect sensitivity.

There are other pathogens that are traditionally detected with serology (e.g., *Rickettsia*, *Bartonella*, *Coxiella*, *Leptospira*, *Brucella*, and hantavirus). Paired serologic testing is intrinsically an imperfect comparator because it audits exposure over a broad time pe-

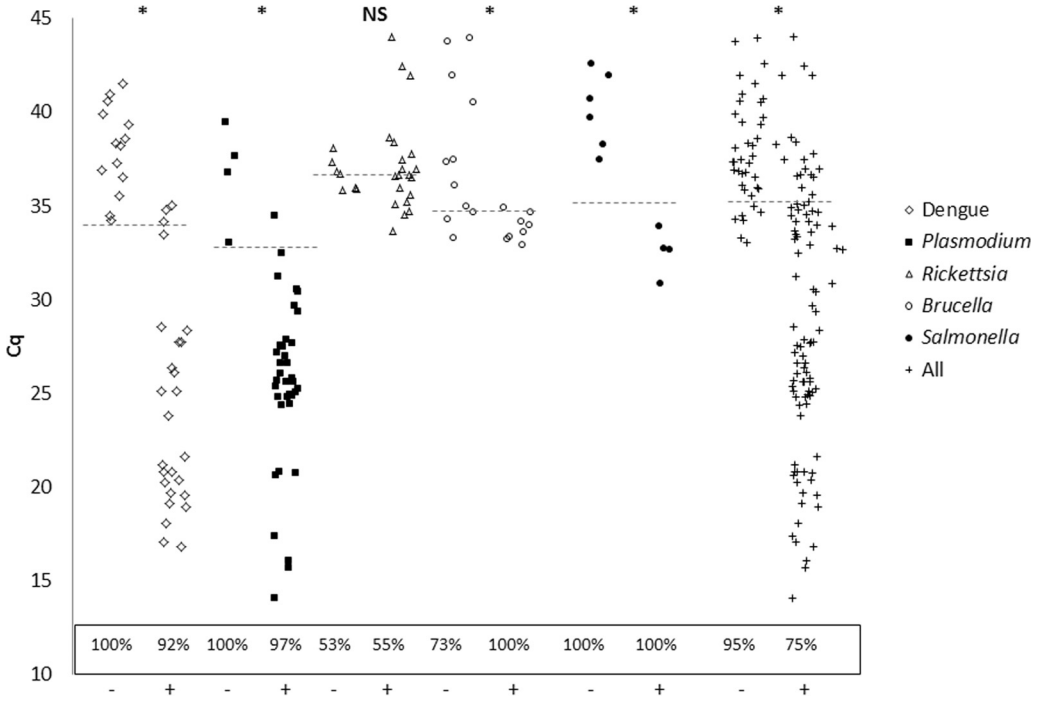

FIG 3 Optimal TAC C_q cutoffs using amplicon sequencing as the reference. The dashed line shows the cutoffs based on ROC analysis. On the *x* axis, symbols indicate that a sample was confirmed by sequencing (+) or not (−). TAC specificity and sensitivity versus sequencing at these cutoffs are shown in the box.

riod while the TAC tests only a single moment (33). Furthermore, these pathogens cause bacteremia only during certain stages of illness due to different pathogeneses. For example, rickettsemia occurs 5 to 10 days after onset of illness, and leptospiremia usually appears before clinical presentation. Thus, if one is particularly interested in such pathogens, testing of sequential specimens or with additional modalities would be sensible. That said, we were able to source 48 specimens that were IRTP positive for these pathogens, and against this standard the TAC exhibited an 83% sensitivity (range, 57% to 100%) and a 97% specificity.

Finally, there were a large number of rare pathogens on our card (e.g., yellow fever and CCHF), which could not be clinically validated through the current study. We presume all of our specimens were negative for these pathogens. For such entities, clinical validation will always be difficult; however, we did document a high specificity of these tests, such that there were seemingly no false positives. Therefore, operationally we would recommend keeping these assays on the card (if one is interested in screening for them) and confirming any TAC positives for such pathogens with other means.

Discrepancies, namely, the false-negative/positive TAC results, were generally seen with lower burden infections and very late C_qs by IRTP or TAC. Whether these IRTP or TAC results are truly positive requires further investigation. We know that many of these would not be confirmable by sequencing. Our future plans are to further validate the assays on newly collected specimens. This will allow us to understand the optimal positioning of TAC in AFI surveillance algorithms and to utilize the assay in the field for outbreak investigations. For the moment, we would advocate using whole blood.

ACKNOWLEDGMENTS

The following reagents were obtained through BEI Resources, NIAID, NIH, as part of the Human Microbiome Project: dengue virus nucleic acid panel, NR-32847; genomic DNA from *Brucella melitensis*, strain 16M (NCTC 10094), NR-2525; genomic DNA from *Yersinia pestis*, strain Nepal516, NR-2720; *Plasmodium falciparum* genomic DNA, *Plasmodium falciparum* 7C46, MRA-172G. We also wish to acknowledge Kenya laboratory personnel who assisted with laboratory work. They include Fredrick Ade, Jeremiah Nyaundi, Victoria Mwende, Shirley Lidechi, Jim Katieno, and Patrick Emojong. We thank Alex Hoffmaster and Rebekah Tiller (CDC, NCEZID) for providing *Brucella* genomic DNA. Collection of samples in Moshi was made possible through support from U.S. National Institutes of Health International Studies on AIDS Associated Coinfections (ISAAC) award U01 AI062563. We thank Martin Schutten and Suzan D. Pas (Erasmus MC, Department of Virology, Rotterdam, The Netherlands) for providing PhHV.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the U.S. Centers for Disease Control and Prevention.

FUNDING INFORMATION

CDC provided funding to Eric Houpt under grant number 200-2013-M-57203. NIH provided funding to Eric Houpt under grant number K24AI102972.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

1. **Hammitt LL, Kazungu S, Morpeth SC, Gibson DG, Mvera B, Brent AJ, Mwarumba S, Onyango CO, Bett A, Akech DO, Murdoch DR, Nokes DJ, Scott JA.** 2012. A preliminary study of pneumonia etiology among hospitalized children in Kenya. Clin Infect Dis **54**(Suppl):S190–S199. http://dx.doi.org/10.1093/cid/cir1071.
2. **Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF, Faruque AS, Zaidi AK, Saha D, Alonso PL, Tamboura B, Sanogo D, Onwuchekwa U, Manna B, Ramamurthy T, Kanungo S, Ochieng JB, Omore R, Oundo JO, Hossain A, Das SK, Ahmed S, Qureshi S, Quadri F, Adegbola RA, Antonio M, Hossain MJ, Akinsola A, Mandomando I, Nhampossa T, Acacio S, Biswas K, O'Reilly CE, Mintz ED, Berkeley LY, Muhsen K, Sommerfelt H, Robins-Browne RM, Levine MM.** 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet **382:**209–222. http://dx.doi.org/10.1016/S0140-6736(13)60844-2.
3. **Prasad N, Sharples KJ, Murdoch DR, Crump JA.** 2015. Community prevalence of fever and relationship with malaria among infants and children in low-resource areas. Am J Trop Med Hyg **93:**178–180. http://dx.doi.org/10.4269/ajtmh.14-0646.
4. **World Health Organization.** 2010. Guidelines for the treatment of malaria, 2nd ed. World Health Organization, Geneva, Switzerland.
5. **Kariuki S.** 2008. Typhoid fever in sub-Saharan Africa: challenges of diagnosis and management of infections. J Infect Dev Ctries **2:**443–447.
6. **Ochieng C, Lutomiah J, Makio A, Koka H, Chepkorir E, Yalwala S, Mutisya J, Musila L, Khamadi S, Richardson J, Bast J, Schnabel D, Wurapa E, Sang R.** 2013. Mosquito-borne arbovirus surveillance at selected sites in diverse ecological zones of Kenya; 2007-2012. Virol J **10:**140. http://dx.doi.org/10.1186/1743-422X-10-140.
7. **Brent AJ, Ahmed I, Ndiritu M, Lewa P, Ngetsa C, Lowe B, Bauni E, English M, Berkley JA, Scott JA.** 2006. Incidence of clinically significant bacteraemia in children who present to hospital in Kenya: community-based observational study. Lancet **367:**482–488. http://dx.doi.org/10.1016/S0140-6736(06)68180-4.
8. **D'Acremont V, Kilowoko M, Kyungu E, Philipina S, Sangu W, Kahama-Maro J, Lengeler C, Cherpillod P, Kaiser L, Genton B.** 2014. Beyond malaria–causes of fever in outpatient Tanzanian children. N Engl J Med **370:**809–817. http://dx.doi.org/10.1056/NEJMoa1214482.
9. **Baize S, Pannetier D, Oestereich L, Rieger T, Koivogui L, Magassouba N, Soropogui B, Sow MS, Keita S, De Clerck H, Tiffany A, Dominguez G, Loua M, Traore A, Kolie M, Malano ER, Heleze E, Bocquin A, Mely S, Raoul H, Caro V, Cadar D, Gabriel M, Pahlmann M, Tappe D, Schmidt-Chanasit J, Impouma B, Diallo AK, Formenty P, Van Herp M, Gunther S.** 2014. Emergence of Zaire Ebola virus disease in Guinea. N Engl J Med **371:**1418–1425. http://dx.doi.org/10.1056/NEJMoa1404505.
10. **Crump JA, Morrissey AB, Nicholson WL, Massung RF, Stoddard RA, Galloway RL, Ooi EE, Maro VP, Saganda W, Kinabo GD, Muiruri C, Bartlett JA.** 2013. Etiology of severe non-malaria febrile illness in Northern Tanzania: a prospective cohort study. PLoS Negl Trop Dis **7:**e2324. http://dx.doi.org/10.1371/journal.pntd.0002324.
11. **Prasad N, Murdoch DR, Reyburn H, Crump JA.** 2015. Etiology of severe febrile illness in low- and middle-income countries: a systematic review. PLoS One **10:**e0127962. http://dx.doi.org/10.1371/journal.pone.0127962.
12. **Towner JS, Pourrut X, Albarino CG, Nkogue CN, Bird BH, Grard G, Ksiazek TG, Gonzalez JP, Nichol ST, Leroy EM.** 2007. Marburg virus infection detected in a common African bat. PLoS One **2:**e764. http://dx.doi.org/10.1371/journal.pone.0000764.
13. **Towner JS, Sealy TK, Khristova ML, Albarino CG, Conlan S, Reeder SA, Quan PL, Lipkin WI, Downing R, Tappero JW, Okware S, Lutwama J, Bakamutumaho B, Kayiwa J, Comer JA, Rollin PE, Ksiazek TG, Nichol ST.** 2008. Newly discovered Ebola virus associated with hemorrhagic fever outbreak in Uganda. PLoS Pathog **4:**e1000212. http://dx.doi.org/10.1371/journal.ppat.1000212.
14. **Diaz MH, Waller JL, Napoliello RA, Islam MS, Wolff BJ, Burken DJ, Holden RL, Srinivasan V, Arvay M, McGee L, Oberste MS, Whitney CG, Schrag SJ, Winchell JM, Saha SK.** 2013. Optimization of multiple pathogen detection using the TaqMan array card: application for a population-based study of neonatal infection. PLoS One **8:**e66183. http://dx.doi.org/10.1371/journal.pone.0066183.
15. **Kodani M, Yang G, Conklin LM, Travis TC, Whitney CG, Anderson LJ, Schrag SJ, Taylor TH, Jr, Beall BW, Breiman RF, Feikin DR, Njenga MK, Mayer LW, Oberste MS, Tondella ML, Winchell JM, Lindstrom SL, Erdman DD, Fields BS.** 2011. Application of TaqMan low-density arrays for simultaneous detection of multiple respiratory pathogens. J Clin Microbiol **49:**2175–2182. http://dx.doi.org/10.1128/JCM.02270-10.
16. **Liu J, Gratz J, Amour C, Kibiki G, Becker S, Janaki L, Verweij JJ, Taniuchi M, Sobuz SU, Haque R, Haverstick DM, Houpt ER.** 2013. A

laboratory-developed TaqMan array card for simultaneous detection of 19 enteropathogens. J Clin Microbiol **51:**472–480. http://dx.doi.org/10.1128/JCM.02658-12.

17. **Liu J, Kabir F, Manneh J, Lertsethtakarn P, Begum S, Gratz J, Becker SM, Operario DJ, Taniuchi M, Janaki L, Platts-Mills JA, Haverstick DM, Kabir M, Sobuz SU, Nakjarung K, Sakpaisal P, Silapong S, Bodhidatta L, Qureshi S, Kalam A, Saidi Q, Swai N, Mujaga B, Maro A, Kwambana B, Dione M, Antonio M, Kibiki G, Mason CJ, Haque R, Iqbal N, Zaidi AK, Houpt ER.** 2014. Development and assessment of molecular diagnostic tests for 15 enteropathogens causing childhood diarrhoea: a multicentre study. Lancet Infect Dis **14:**716–724. http://dx.doi.org/10.1016/S1473-3099(14)70808-4.
18. **Summers A, Nyenswah TG, Montgomery JM, Neatherlin J, Tappero, Centers for Disease Control and Prevention.** 2014. Challenges in responding to the Ebola epidemic - four rural counties, Liberia, August-November 2014. MMWR Morb Mortal Wkly Rep **63:**1202–1204.
19. **Kodani M, Winchell JM.** 2012. Engineered combined-positive-control template for real-time reverse transcription-PCR in multiple-pathogen-detection assays. J Clin Microbiol **50:**1057–1060. http://dx.doi.org/10.1128/JCM.05987-11.
20. **Towner JS, Khristova ML, Sealy TK, Vincent MJ, Erickson BR, Bawiec DA, Hartman AL, Comer JA, Zaki SR, Stroher U, Gomes Da Silva F, del Castillo F, Rollin PE, Ksiazek TG, Nichol ST.** 2006. Marburgvirus genomics and association with a large hemorrhagic fever outbreak in Angola. J Virol **80:**6497–6516. http://dx.doi.org/10.1128/JVI.00069-06.
21. **Imwong M, Woodrow CJ, Hendriksen IC, Veenemans J, Verhoef H, Faiz MA, Mohanty S, Mishra S, Mtove G, Gesase S, Seni A, Chhaganlal KD, Day NP, Dondorp AM, White NJ.** 2015. Plasma concentration of parasite DNA as a measure of disease severity in falciparum malaria. J Infect Dis **211:**1128–1133. http://dx.doi.org/10.1093/infdis/jiu590.
22. **Reddy V, Mani RS, Desai A, Ravi V.** 2014. Correlation of plasma viral loads and presence of Chikungunya IgM antibodies with cytokine/chemokine levels during acute Chikungunya virus infection. J Med Virol **86:**1393–1401. http://dx.doi.org/10.1002/jmv.23875.
23. **Towner JS, Rollin PE, Bausch DG, Sanchez A, Crary SM, Vincent M, Lee WF, Spiropoulou CF, Ksiazek TG, Lukwiya M, Kaducu F, Downing R, Nichol ST.** 2004. Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. J Virol **78:**4330–4341. http://dx.doi.org/10.1128/JVI.78.8.4330-4341.2004.
24. **Wang WK, Chen HL, Yang CF, Hsieh SC, Juan CC, Chang SM, Yu CC, Lin LH, Huang JH, King CC.** 2006. Slower rates of clearance of viral load and virus-containing immune complexes in patients with dengue hemorrhagic fever. Clin Infect Dis **43:**1023–1030. http://dx.doi.org/10.1086/507635.
25. **de Oliveira Poersch C, Pavoni DP, Queiroz MH, de Borba L, Goldenberg S, dos Santos CN, Krieger MA.** 2005. Dengue virus infections: comparison of methods for diagnosing the acute disease. J Clin Virol **32:**272–277. http://dx.doi.org/10.1016/j.jcv.2004.08.008.
26. **Gyarmati P, Mohammed N, Norder H, Blomberg J, Belak S, Widen F.** 2007. Universal detection of hepatitis E virus by two real-time PCR assays: TaqMan and primer-probe energy transfer. J Virol Methods **146:**226–235. http://dx.doi.org/10.1016/j.jviromet.2007.07.014.
27. **Vasoo S, Pritt BS.** 2013. Molecular diagnostics and parasitic disease. Clin Lab Med **33:**461–503. http://dx.doi.org/10.1016/j.cll.2013.03.008.
28. **Towner JS, Sealy TK, Ksiazek TG, Nichol ST.** 2007. High-throughput molecular detection of hemorrhagic fever virus threats with applications for outbreak settings. J Infect Dis **196**(Suppl)**:**S205–S212. http://dx.doi.org/10.1086/520601.
29. **Chang SS, Hsieh WH, Liu TS, Lee SH, Wang CH, Chou HC, Yeo YH, Tseng CP, Lee CC.** 2013. Multiplex PCR system for rapid detection of pathogens in patients with presumed sepsis - a systemic review and meta-analysis. PLoS One **8:**e62323. http://dx.doi.org/10.1371/journal.pone.0062323.
30. **Gordon MA, Kankwatira AM, Mwafulirwa G, Walsh AL, Hopkins MJ, Parry CM, Faragher EB, Zijlstra EE, Heyderman RS, Molyneux ME.** 2010. Invasive non-typhoid salmonellae establish systemic intracellular infection in HIV-infected adults: an emerging disease pathogenesis. Clin Infect Dis **50:**953–962. http://dx.doi.org/10.1086/651080.
31. **Wain J, Pham VB, Ha V, Nguyen NM, To SD, Walsh AL, Parry CM, Hasserjian RP, HoHo VA, Tran TH, Farrar J, White NJ, Day NP.** 2001. Quantitation of bacteria in bone marrow from patients with typhoid fever: relationship between counts and clinical features. J Clin Microbiol **39:**1571–1576. http://dx.doi.org/10.1128/JCM.39.4.1571-1576.2001.
32. **Baker S, Favorov M, Dougan G.** 2010. Searching for the elusive typhoid diagnostic. BMC Infect Dis **10:**45. http://dx.doi.org/10.1186/1471-2334-10-45.
33. **Schotthoefer AM, Meece JK, Ivacic LC, Bertz PD, Zhang K, Weiler T, Uphoff TS, Fritsche TR.** 2013. Comparison of a real-time PCR method with serology and blood smear analysis for diagnosis of human anaplasmosis: importance of infection time course for optimal test utilization. J Clin Microbiol **51:**2147–2153. http://dx.doi.org/10.1128/JCM.00347-13.
34. **Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, Lambert AJ, Campbell GL.** 2007. Chikungunya virus in US travelers returning from India, 2006. Emerg Infect Dis **13:**764–767. http://dx.doi.org/10.3201/eid1305.070015.
35. **Drosten C, Gottig S, Schilling S, Asper M, Panning M, Schmitz H, Gunther S.** 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J Clin Microbiol **40:**2323–2330. http://dx.doi.org/10.1128/JCM.40.7.2323-2330.2002.
36. **Kramski M, Meisel H, Klempa B, Kruger DH, Pauli G, Nitsche A.** 2007. Detection and typing of human pathogenic hantaviruses by real-time reverse transcription-PCR and pyrosequencing. Clin Chem **53:**1899–1905. http://dx.doi.org/10.1373/clinchem.2007.093245.
37. **Jothikumar N, Cromeans TL, Robertson BH, Meng XJ, Hill VR.** 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J Virol Methods **131:**65–71. http://dx.doi.org/10.1016/j.jviromet.2005.07.004.
38. **Lo MK, Lowe L, Hummel KB, Sazzad HM, Gurley ES, Hossain MJ, Luby SP, Miller DM, Comer JA, Rollin PE, Bellini WJ, Rota PA.** 2012. Characterization of Nipah virus from outbreaks in Bangladesh, 2008-2010. Emerg Infect Dis **18:**248–255. http://dx.doi.org/10.3201/eid1802.111492.
39. **Saxton-Shaw KD, Ledermann JP, Borland EM, Stovall JL, Mossel EC, Singh AJ, Wilusz J, Powers AM.** 2013. O'nyong nyong virus molecular determinants of unique vector specificity reside in non-structural protein 3. PLoS Negl Trop Dis **7:**e1931. http://dx.doi.org/10.1371/journal.pntd.0001931.
40. **Bird BH, Bawiec DA, Ksiazek TG, Shoemaker TR, Nichol ST.** 2007. Highly sensitive and broadly reactive quantitative reverse transcription-PCR assay for high-throughput detection of Rift Valley fever virus. J Clin Microbiol **45:**3506–3513. http://dx.doi.org/10.1128/JCM.00936-07.
41. **Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, Komar N, Panella NA, Allen BC, Volpe KE, Davis BS, Roehrig JT.** 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol **38:**4066–4071.
42. **Chao DY, Davis BS, Chang GJ.** 2007. Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes. J Clin Microbiol **45:**584–589. http://dx.doi.org/10.1128/JCM.00842-06.
43. **Diaz MH, Bai Y, Malania L, Winchell JM, Kosoy MY.** 2012. Development of a novel genus-specific real-time PCR assay for detection and differentiation of *Bartonella* species and genotypes. J Clin Microbiol **50:**1645–1649. http://dx.doi.org/10.1128/JCM.06621-11.
44. **Hinic V, Brodard I, Thomann A, Cvetnic Z, Makaya PV, Frey J, Abril C.** 2008. Novel identification and differentiation of *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* suitable for both conventional and real-time PCR systems. J Microbiol Methods **75:**375–378. http://dx.doi.org/10.1016/j.mimet.2008.07.002.
45. **Loftis AD, Reeves WK, Szumlas DE, Abbassy MM, Helmy IM, Moriarity JR, Dasch GA.** 2006. Surveillance of Egyptian fleas for agents of public health significance: *Anaplasma*, *Bartonella*, *Coxiella*, *Ehrlichia*, *Rickettsia*, and *Yersinia pestis*. Am J Trop Med Hyg **75:**41–48.
46. **Stoddard RA, Gee JE, Wilkins PP, McCaustland K, Hoffmaster AR.** 2009. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. Diagn Microbiol Infect Dis **64:**247–255. http://dx.doi.org/10.1016/j.diagmicrobio.2009.03.014.
47. **Kato CY, Chung IH, Robinson LK, Austin AL, Dasch GA, Massung RF.** 2013. Assessment of real-time PCR assay for detection of *Rickettsia* spp. and *Rickettsia rickettsii* in banked clinical samples. J Clin Microbiol **51:**314–317. http://dx.doi.org/10.1128/JCM.01723-12.
48. **Malorny B, Cook N, D'Agostino M, De Medici D, Croci L, Abdulmawjood A, Fach P, Karpiskova R, Aymerich T, Kwaitek K, Hoorfar J,**

Malorny B. 2004. Multicenter validation of PCR-based method for detection of *Salmonella* in chicken and pig samples. J AOAC Int **87:**861–866.
49. **Nga TV, Karkey A, Dongol S, Thuy HN, Dunstan S, Holt K, Tu le TP, Campbell JI, Chau TT, Chau NV, Arjyal A, Koirala S, Basnyat B, Dolecek C, Farrar J, Baker S.** 2010. The sensitivity of real-time PCR amplification targeting invasive *Salmonella* serovars in biological specimens. BMC Infect Dis **10:**125. http://dx.doi.org/10.1186/1471-2334-10-125.
50. **Wortmann G, Sweeney C, Houng HS, Aronson N, Stiteler J, Jackson J, Ockenhouse C.** 2001. Rapid diagnosis of leishmaniasis by fluorogenic polymerase chain reaction. Am J Trop Med Hyg **65:**583–587.
51. **Kamau E, Tolbert LS, Kortepeter L, Pratt M, Nyakoe N, Muringo L, Ogutu B, Waitumbi JN, Ockenhouse CF.** 2011. Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for detection and quantitation of plasmodium by amplifying RNA and DNA of the 18S rRNA genes. J Clin Microbiol **49:**2946–2953. http://dx.doi.org/10.1128/JCM.00276-11.
52. **Deborggraeve S, Claes F, Laurent T, Mertens P, Leclipteux T, Dujardin JC, Herdewijn P, Buscher P.** 2006. Molecular dipstick test for diagnosis of sleeping sickness. J Clin Microbiol **44:**2884–2889. http://dx.doi.org/10.1128/JCM.02594-05.